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Crosslinking Efficiency of Gelatin Hardeners*

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Synopsis

By using the criteria of melting point and sol fraction, a study has been made of the stoichiometric efficiencies of hardeners for gelatin, as distinct from their rates of reaction. The factors which affect these measurements were investigated. Both melting point and sol fraction distinguish gelatins in respect of molecular weight and origin. Sol fraction measurements give evidence of molecular weight distribution. Various hardeners have a wide range of molecular efficiencies, and these do not correlate with rates of reaction as judged from solution viscosity rise methods. Sol fraction measurements can separate hardeners which would have been considered similar from melting point behavior.

INTRODUCTION

The hardening of gelatin is a process, similar to the tanning of leather, which toughens wet gelatin, making it more resistant to abrasion. It reduces the swelling in water and increases the temperature required for solution in water, the so-called melting point. Hardening is a result of chemical crosslinking between the gelatin and the hardening agent.[1,2]

Many of the ideas as to the nature of the crosslinking reactions involved in the hardening of gelatin come from the investigations of collagen.[3] Gelatin itself has received some attention in recent years,[4-6] but this has been principally kinetic studies concerned with rates of crosslinking in solution.

Measurements on hardened gelatin layers have been used for practical purposes.[7] Apart from some work on elastic properties,[8] there has been little attempt to correlate these measurements with fundamental properties. This is partly because the term "hardening" is applied to the effects produced on a wide range of gelatin properties (solution viscosity and setting, melting point, swelling, resistance to mechanical abrasion, etc.), which are not simply interrelated. Also, the methods of measurement generally used gave results not readily interpreted in molecular terms.

This paper describes techniques for the measurement of melting point and soluble fraction which define these two aspects of the hardening of gelatin layers. It also shows how they are related to the properties of

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gelatin and hardener. These measurements were made on gelatin layers formed, with various hardener concentrations, under conditions which are believed to allow the more active hardeners to react completely. The so-called melting point is, in fact, the temperature of dissolution of the layer in water. This is, to some extent, arbitrarily controlled by the conditions of determination. Provided that these conditions are defined, the melting point is a measure of the strength of the crosslinked network. It is related to the number of crosslinks introduced per gelatin molecule and their thermal stability when this is less than that of the main chains. The amount of a layer which is still soluble after crosslinking (the sol fraction as distinct from the gel network) is an analytically defined quantity whose physical chemical significance has been studied theoretically and in many actual crosslinked systems.[9,10]

EXPERIMENTAL

Formation of Layers

For the work involved in developing these methods, mucochloric acid was used as a convenient solid, relatively nonvolatile hardener.[2,6] Except for experiments in which gelatin concentration was the variable being studied, the layers were prepared by drying down 12% gelatin solutions. Eight 3-g samples of gelatin were each soaked in 21 ml of distilled water and then dispersed at 40°C. The hardener was introduced from a suitable solution by a range of additions up to 4 ml in volume. For additions less than 4 ml, solvent corresponding to the difference was added to give the same final gelatin concentration in each case. The solutions were adjusted to pH 6.5 (±0.2) after the hardener addition.

The layers were formed by drying down 10 ml from each of the gelatin solutions in straight-sided, stainless-steel dishes of 2 cm diameter (silicone-treated moisture determination dishes, BS 757: 1959, were found to be convenient). This was carried out at 50°C over a period of 18 hr, with the dishes resting on a leveled platform at the bottom of an oven. The oven was equipped with galleries containing calcium chloride to absorb the moisture during the drying, and an efficient circulating fan. The extent of reaction of the hardener depends on the time of drying and on the temperature. These depend, in turn, on the initial volume of gelatin solution, the size of the oven, and the amount of calcium chloride used. The amount of calcium chloride taken was adjusted to obtain the highest possible melting point (see below) for a given hardener (mucochloric acid) addition with the particular oven used (Fig. 1). This condition was taken to represent the most complete reaction.

This is supported by the fact that, although mucochloric acid-containing layers prepared by rapid evaporation on a steam bath had lower melting points than those prepared in the oven, repeated wetting and redrying brought the melting points of these layers up close to those obtained directly in the oven. Also, layers prepared in the oven at pH's 5.5, 6.5,

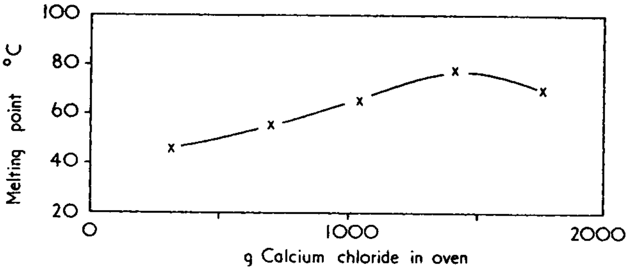

Fig. 1. Variation of melting point obtained from a given mucochloric acid addition with various amounts of calcium chloride in the oven during the preparation of the layers.

and 7.0 gave the same melting points, and sol-fraction experiments with samples which had been kept 18 days after preparation and grinding gave the same values as obtained by immediate extraction.

The layers were readily separated from the treated stainless-steel dishes and were then used for measurements of melting point and sol fraction.

Melting Point Determination

The melting points, in water, were taken as the temperature of rupture of the swollen gelatin layer by the weight of a $^5/_{32}$-in. stainless-steel ball bearing.

A piece of the gelatin layer was imprisoned between two pieces of 0.008-in. stainless-steel shim, each of which was pierced by a $^1/_4$-in. diameter hole. This sandwich, with the holes brought into coincidence, was slipped between the turns of a spring holder. Sixteen of these (duplicate measurements on the eight separate layers) were mounted horizontally on a circular support in a 2-liter beaker of distilled water and allowed to soak for $^3/_4$ hr at room temperature. The ball bearings were placed in the cups formed by the swelling of the gelatin and soaking was continued for a further $^1/_4$ hr. This part of the apparatus is shown in Figure 2. The temperature was then raised by 1°C/min by a hot plate. The water was stirred at 5 rpm by a multibladed paddle of large diameter which virtually covered the bottom of the beaker. This gave a uniform temperature distribution without the layers being mechanically disturbed by the agitation. With small amounts of hardening, the disintegration of the layer is quite sudden. With increased amounts, however, considerable extension occurred but the melting point was still quite sharp at the temperature of final rupture of the layer. It was found that, provided the layers were fully swollen, the melting points were not particularly sensitive to the rate of heating ($\pm 10\%$) or affected by $\pm 10\%$ variation in thickness.

Sol-Fraction Determination

To provide sufficient material for convenient handling, two layers at each hardener concentration were used. These were ground (e.g., in a

Moulinex grinder or a Waring Blendor) to a particle size between 20 and 60 mesh. The particle-size range used for sol-fraction extraction was determined by experiment. This was a compromise between more rapid extraction with smaller particles and the difficulty of separating these by filtration when they were partly dissolved. A weighed sample was extracted with water at 40°C for 3 hr ($^1/_2$ g with 50 ml of distilled water in a 75-ml capacity glass-stoppered tube; the contents were mixed by inversion at 15-min intervals). A sample of the solution was separated from any remaining gel fraction by filtration and analyzed by the biuret method[11]

Fig. 2. Assembly for supporting layers during melting point determination.

to determine the sol fraction. Allowance was made for the moisture content of the layers by a blank experiment without hardener.

At all levels of crosslinking tried, the amount extracted rose sharply within the first hour and remained substantially constant between 2 and 4 hr (Fig. 3). After this there was a slow increase, presumably due to the breakdown of the gel network. The value at 3 hr was, therefore, taken as the sol fraction, and this was not affected by increased solvent volume or successive extractions. The hydrogen-bond-breaking solvent, 1*M* potassium thiocyanate, was also tried. The amount extracted was almost the same as with water. It was, if anything, slightly lower.

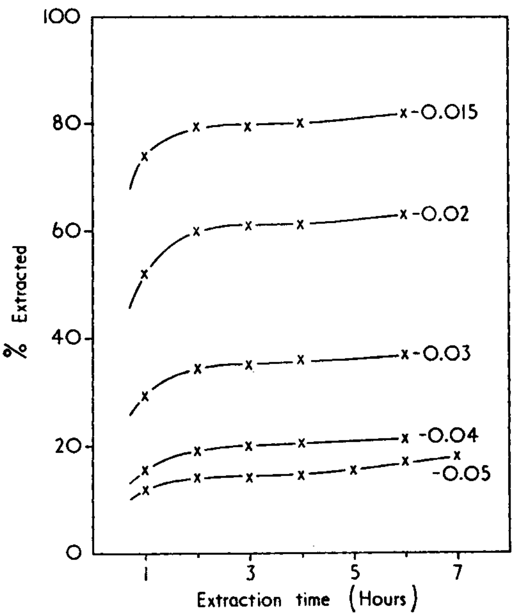

Fig. 3. Variation with time of amount extracted at different levels of hardening (indicated as mmole mucochloric acid per g gelatin).

RESULTS

Since it is the stoichiometry of the hardening process which is being considered, the concentrations are expressed as millimole hardener per gram of gelatin. The melting point results are usually given as simple plots against hardener concentration. Theoretical studies[12] indicate that the shape of log plots of sol fraction (S) against hardener concentration ([H]) should be related to the initial molecular weight distribution of the gelatin. It has also been pointed out,[9,10] that a particularly useful way to analyze data is to plot ($S + S^{1/2}$) against $[\mathrm{H}]^{-1}$. Both of these plots are used where appropriate.

Dependence on Initial Gelatin Concentration

For a given hardener concentration, the melting point increases with initial gelatin concentration up to a limiting value at about 12% (Fig. 4*a*). The variation is greatest for layers with lower hardener concentrations. Similarly, the sol fraction decreases towards a limiting value at about the same 12% initial gelatin concentration (Fig. 4*b*). Over the wide range of hardener concentrations used, initial gelatin concentrations greater than 12% did not measurably increase the hardening (crosslinking) as determined by these methods (Fig. 5).

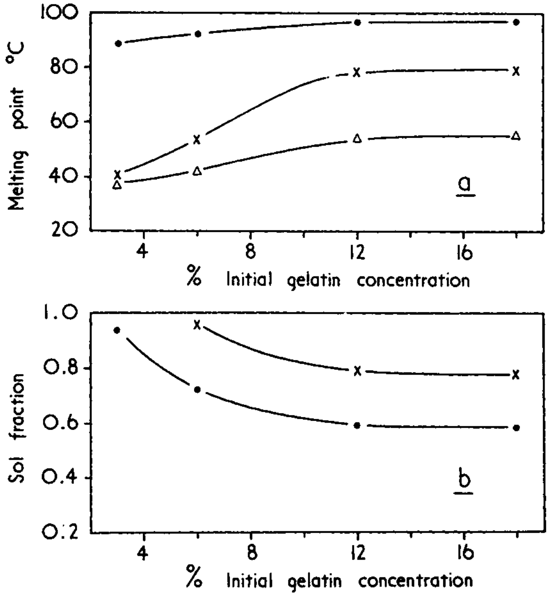

Fig. 4. Effect of initial gelatin concentration on (*a*) the melting point and (*b*) sol fraction of layers hardened with mucochloric acid: (●) 0.02 mmole; (×) 0.015 mmole; (Δ) 0.01 mmole/g gelatin.

Relation of Melting Point and Sol Fraction

Sol-fraction measurements can indicate higher levels of hardening than are readily accessible to melting-point determination (Fig. 6). For the example shown, the layer with sufficient hardening to remain intact at the boiling point of water still contains some 40% of its gelatin which could be extracted at 40°C.

Dependence on Gelatin

Melting point against hardener concentration curves for different gelatins follow the same general shape, although the effect of a given addition varies (Fig. 7). The viscosities of gelatin solutions measured under standard conditions can be taken as an indication of the relative average molecu-

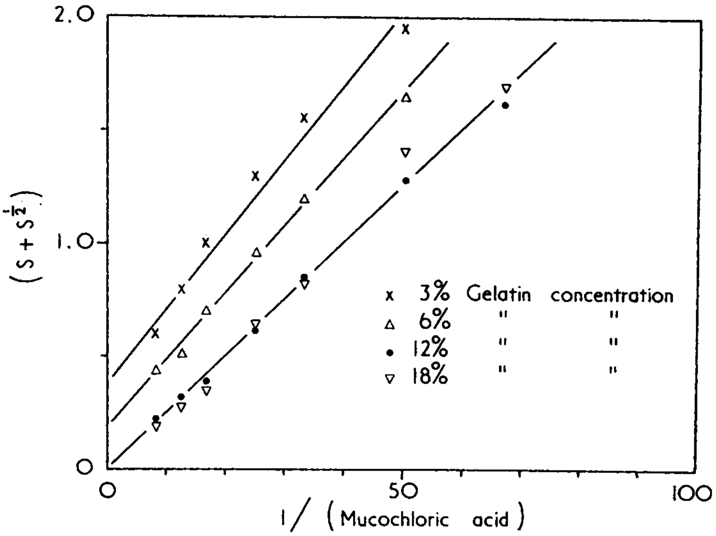

Fig. 5. Effect of initial gelatin concentration on the sol fraction from layers prepared from the same gelatin with a wide range of hardener additions.

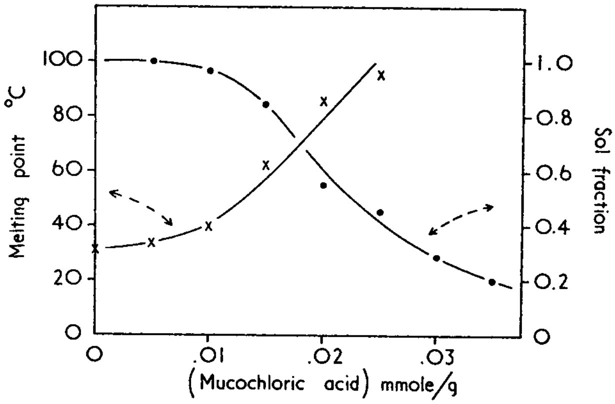

Fig. 6. Variation of (×) melting point and (●) sol fraction with hardener concentration for layers prepared from the same gelatin.

lar weights. For gelatins of each type of manufacture, the amount of hardener required to give a melting point rise to 100°C depends on the molecular weight, as indicated by the viscosity (Fig. 8).

Sol-fraction measurements were made on three pairs of gelatins for which the details of manufacture were known (Fig. 9). These were all single extracts, two each from alkali-processed hide, alkali-processed bone, and acid-processed pigskin. At the lower levels of hardening, the hardening as measured by sol fraction was again related to the gelatin solution viscosity. However, with all three pairs, the later-extract gelatin responded less rapidly to increased hardener concentration, indicating a broader molecular weight distribution.

The effect of broad molecular weight distribution was again apparent in the departure from linearity of $(S + S^{1/2})$ against $1/[H]$ plots for later extract gelatins and lower-quality gelatins of mixed origin (Fig. 10).

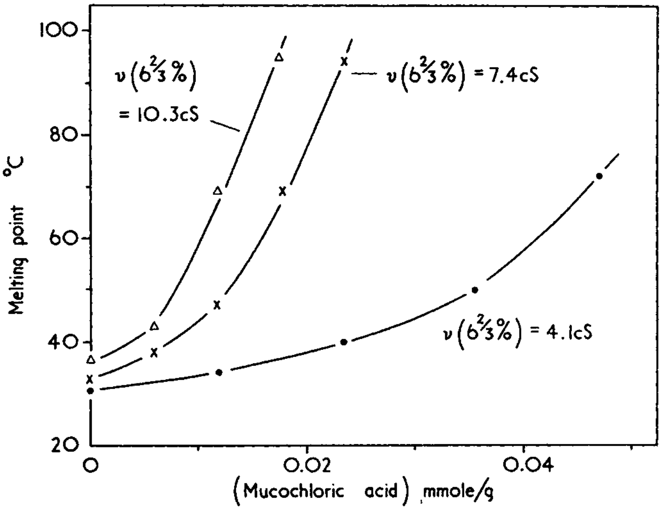

Fig. 7. Variation of melting point with hardener concentration for three gelatins of different molecular weight as indicated by their solution viscosity ν, measured at $6^2/_3\%$ concentration and 40°C.

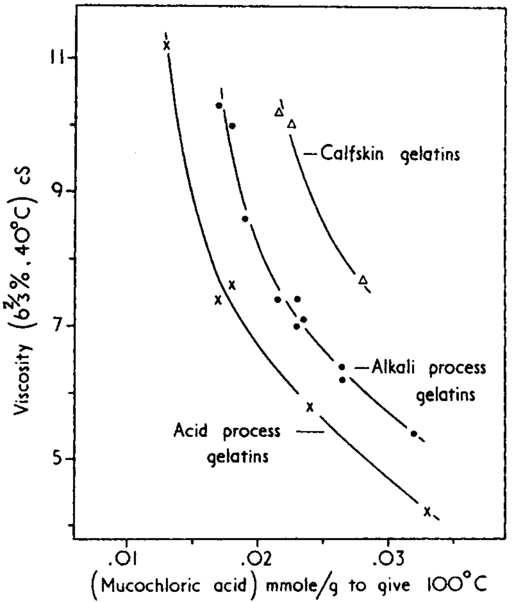

Fig. 8. Dependence on solution viscosity of gelatin hardenability, as shown by the amount of hardener required to give a melting point rise to 100°C.

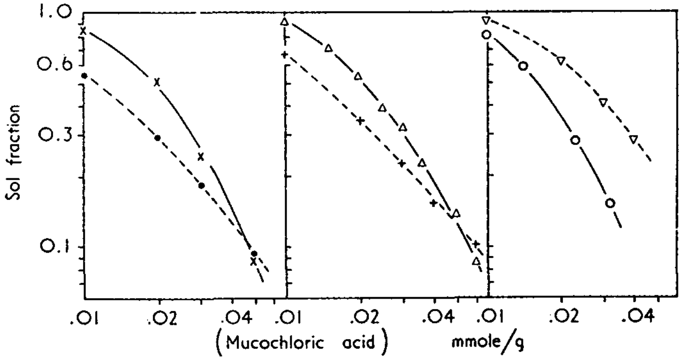

Fig. 9. Variation of sol fraction with hardener concentration for single-extract gelatins: (×) first-extract alkali-processed hide, ν ($6^2/_3\%$, 40°C) = 8.0 cS; (●) second-extract alkali-processed hide, ν ($6^2/_3\%$, 40°C) = 10.7 cS; (Δ) first-extract alkali-processed bone, ν ($6^2/_3\%$, 40°C) = 7.6 cS; (+) fourth-extract alkali-processed bone, ν ($6^2/_3\%$, 40°C) = 9.0 cS; (O) first-extract acid-processed pigskin, ν ($6^2/_3\%$, 40°C) = 9.5 cS; (∇) second-extract acid-processed pigskin, ν ($6^2/_3\%$, 40°C) = 5.9 cS.

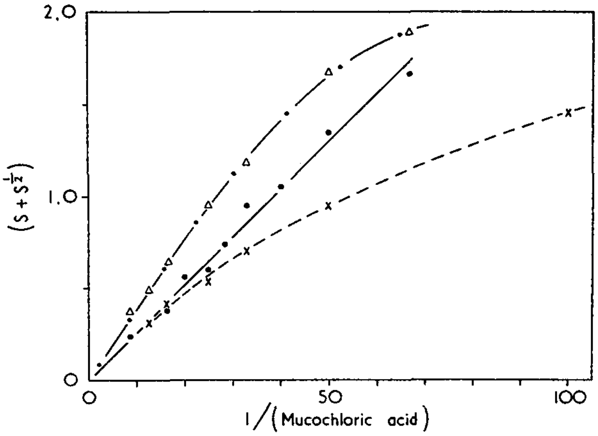

Fig. 10. Dependence of the sol fraction/hardener concentration relation on gelatin extraction and quality, Charlesby-Pinner plot: (●) first-extract alkali-processed bone, ν ($6^2/_3\%$, 40°C) = 7.6 cS; (×) fourth-extract alkali-processed bone, ν ($6^2/_3\%$, 40°C) = 9.0 cS; (Δ) low-quality alkali-process gelatin of blended extracts, ν ($6^2/_3\%$, 40°C) = 5.1 cS.

Dependence on Hardener

The great majority of hardeners gave melting-point curves similar in general shape to those for different gelatins. With a "standard" gelatin, the molecular efficiency, as judged by the concentration of hardener required to produce a given melting point, varied widely. Examples are given in Table I, together with relative rates of reaction where these had been measured. (The rates of reaction were obtained by a solution viscosity-rise technique.[5] For the purpose of comparison, these were calcu-

TABLE I
Hardening Efficiencies and Rates for Various Hardeners

Hardening agent	Hardener to give 100°C melting point, mmole/g gelatin	Rate of hardening, relative to that of formaldehyde, at 0.025M, pH 6.6, 10% gelatin
Formaldehyde	≈0.025 estimated[a]	1
Mucochloric acid	0.022	Too slow to be measured
1,3-Difluoro-4,6-dinitrobenzene	0.022	6
Glutaraldehyde	0.024	3,600
2-Methylglutaraldehyde	0.025	70
Glyoxal	0.049	0.4
2,3-Dihydroxy-1,4-dioxane	0.045	0.3

[a] The oven method for preparing layers cannot be used for volatile hardeners, and this estimate was obtained from gelatin layers sealed so that the formaldehyde could not escape and held for a long period.[13]

lated to a common set of concentrations from a knowledge of the kinetics of the individual hardening agents.)

In general, sol-fraction measurements for different hardeners with a "standard" gelatin gave the same order of efficiencies as obtained from the melting points (Fig. 11). However, 1,3-difluoro-4,6-dinitrobenzene (DF-DNB), for example, an agent which on melting-point data would have been

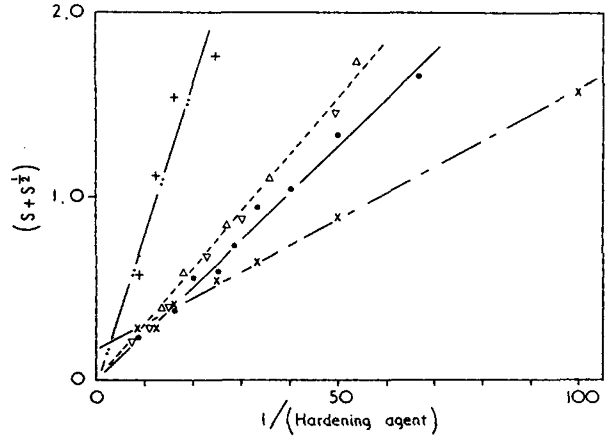

Fig. 11. Dependence of sol fraction of a given gelatin on hardener concentration for various hardeners: (+) glyoxal; (△) glutaraldehyde; (▽) 2-methylglutaraldehyde; (●) mucochloric acid; (×) 1,3-difluoro-4,6-dinitrobenzene.

judged very similar to mucochloric acid, gave a very different sol-fraction curve, indicating that some complication of the reaction was involved.

DISCUSSION

For mucochloric acid the melting points of layers obtained by the oven technique of preparation appeared independent of all factors except the hardener concentration. Similarly, the sol fraction from these layers appeared to be a minimum for any given mucochloric acid addition. Of a great many hardeners investigated by these methods a few have equalled, but none have surpassed, mucochloric acid in terms of molecular efficiency as judged by the number of mmole required per gram of gelatin to produce a given melting-point rise. This all suggests that the method of preparation achieves complete reaction of the more active hardeners.

This is further supported by the quantitative relation of these more active agents to the molecular weight of the gelatin. For any molecular weight distribution it has been shown that at the gel point there will be one crosslinked unit per "weight-average" molecule.[14] The determination of the gel-point concentration for the added hardener (i.e., that concentration of crosslinking agent which just produces a continuous gel) is made more difficult by the presence of the naturally occurring thermally dependent gelling mechanism. It will however be approximately the concentration required to keep the layer intact for the smallest temperature rise which would be expected to eliminate the natural gelling forces. For most gelatins these forces would be considered as largely inoperative at 40°C. This is supported by the similarity of the sol fractions extracted at 40°C by both water and the hydrogen-bond-breaking solvent, $1M$ potassium thiocyanate. For the gelatin used for much of this work, which has a weight-average molecular weight ($\bar{M}_w$) by scattered light measurements of about 2×10^5, the concentration of mucochloric acid required to give a melting point of 40°C was 0.0065 mmole/g. This is only slightly greater than the amount expected from $\bar{M}_w$, 0.005 mmole/g. This indicates the formation of effective crosslinks by the great majority of the hardener added.

Although the hardener concentration to give a 40°C melting point is probably most closely related to the gel-point concentration, because of the low slope of the curves at this level of hardening it is not easy to determine with any precision. On the other hand, the concentration required to give a layer just insoluble in boiling water is more readily determined and is still at a sufficiently low level of crosslinking for it to be expected to follow the same sort of dependence on molecular weight. The hardening ability of various gelatins, as judged from the concentration of mucochloric acid required to give a melting point of 100°C, depends on the gelatin solution viscosity, provided that the gelatins are divided into types of manufacture (Fig. 8). The solution viscosity is a reasonable indication of relative molecular weights so that this relation, within a particular type of gelatin, is to be expected. The different relations for different types of gelatins are similar to those previously observed for hardening rates.[5] Since the gel-point relation is independent of molecular weight distribution, it seems likely that the differences in reactivity between the gelatin types must be due to chemical causes.

The variation of both melting point and sol fraction at low initial gelatin concentrations (Figs. 4*a* and 4*b*) may be attributed to the variation in wastage by intramolecular linking which has been shown to be an important factor in hardening and dependent on gelatin concentration.[15,16] The independence of initial gelatin concentration for both melting point and sol fraction above 12% shows that, either wastage of this type is no longer significant or if intramolecular bonding is still occurring it is of a type inherent to the molecules and not significantly affected by further concentration of the gelatin. The slopes of $(S + S^{1/2})$ against $1/[\mathrm{H}]$ plots for sol-fraction data from layers formed at different initial gelatin concentrations (Fig. 5) show the increasing efficiency with concentration up to 12%. They also show by the intercept at infinite hardening-agent concentration (chain scission parameter) that under the conditions of formation at lower gelatin concentration, a small amount of degradation was occurring. This is presumably due to the longer period for which the gelatin remains as a more dilute solution whilst drying down. This is absent with initial gelatin concentrations of 12% and above and with mucochloric acid the curves pass through the origin.

The less steeply sloped curves of log sol fraction plotted against log hardener concentration for the later extract gelatins (Fig. 9) indicate that these are of broader molecular weight distribution. This agrees with Pouradier's observations.[17] The shapes of these curves over most of the hardener range agree quite well with those predicted by Scott[12] and indicate $\overline{M}_n/\overline{M}_w$ values in the range 1.5–4. They tend, however, to be more curved at low levels of crosslinking than is predicted by this theory.

The $(S + S^{1/2})$ against $1/[\mathrm{H}]$ plots for first-extract and high-quality gelatins (Figs. 5 and 10) are, within the limits of experimental error, linear over most of the hardener concentration range. This behavior was predicted by Charlesby and Pinner[9] for polymers with an exponential type of distribution. This type of distribution is also the basic assumption of the theory given by Scott.[12]

There is, however, a tendency at very low hardening levels for the plots to become more curved toward the $1/[\mathrm{H}]$ axis, and this becomes more clearly marked with later-extract and poorer-quality gelatins. This type of behavior was predicted for broad distributions which were not of a systematic exponential type[9] and has been demonstrated practically.[10] This all suggests that the molecular weight distributions of gelatins tend to have a high molecular weight tail which is important at the low levels of hardening of the gel-point concentration. This would explain why the apparent gel-point concentrations obtained by extrapolation from higher levels of crosslinking are 4–5 times greater than expected from the molecular weight of the gelatins and from those observed on the basis of the 40°C criterion.

Although melting-point criteria divide gelatin hardenability into the same manufacturing types as do viscometric hardening rates, there is no simple relation between hardening rate and stoichiometric hardener efficiency (Table I). Thus mucochloric acid, with a rate of reaction too slow to be meaningfully measured by the viscosity rise technique, is one of the most efficient known. Again steric factors can sometimes affect the hardening rate and with other systems affect neither the rate nor the efficiency (cf. glutaraldehyde and 2-methylglutaraldehyde, also glyoxal and its ethylene glycol adduct 2,3-dihydroxy-1,4-dioxane).

In general, the slopes of the $(S + S^{1/2})$ against $1/[\mathrm{H}]$ plots for different hardeners (Fig. 11) indicate the same order of efficiencies as observed from melting points. With 1,3-difluoro-4,6-dinitrobenzene, an agent which on melting-point data would have been judged very similar to mucochloric acid (Table I), the sol-fraction curves (Fig. 11) show that there are complicating reactions at high levels of hardening.

From this discussion it can be concluded that the crosslinking efficiency of gelatin hardeners is affected by a variety of factors dependent on both the gelatins and the various hardeners. The melting point and sol-fraction techniques described have proved useful in determining some of these.

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